tions has already been pointed out by Bessey, Lowry & Love (1949) and Weber (1950). However, since the quenching of fluorescence is constant, fluorescence measurements can be used, with an error of ± 2 %, for the determination of the eluted flavins. Fluorimetry has been preferred, as it is 30 times more sensitive than spectrophotometry at 260 mμ. and 100 times more sensitive than colorimetry (Hegsted, 1954).

The separation of larger amounts of each compound has been obtained by using cellulose-column electrophoresis; this method can therefore be employed for micropreparative purposes.

A differential adsorption on cellulose of some flavins has been observed. As will be seen from the elution curves in Fig. 4, the degree of adsorption increases in the following order: FAD (=FMN), riboflavin, lumiflavin, lumichrome. Consequently, when displaced 15 cm. down the column before starting the electrophoresis, the flavin mixture undergoes a partial resolution, its components being distributed in distinct zones. When the current is applied the flavins are at different levels in the column and so start their electrophoretic run from different positions. This fact does not hinder the electrophoretic separation on a cellulose column; however, the distribution pattern of the separated compounds is different from that obtained on paper, where all the substances start from the same place.

The methods described here have been successfully applied to the determination of flavins in biological materials.

SUMMARY

A method for the separation and quantitative determination of riboflavin and its derivatives (flavin mononucleotide, flavin-adenine dinucleotide, lumichrome and lumiflavin) by paper electrophoresis is described. A micropreparative separation of the same compounds has been achieved by means of cellulose-column electrophoresis.

The work was aided by grants from the Rockefeller Foundation, to which grateful acknowledgement is made. We also wish to thank Mr P. L. Ipata for skilled technical assistance.

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The Relation between Thiamine, Biotin and Tryptophan Metabolism, Studied in the Rat

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Previous papers (Dalgliesh, 1952; Charconnet-Harding, Dalgliesh & Neuberger, 1953) have shown that useful information on the relationship of B-vitamins to tryptophan metabolism may be obtained by examining urinary metabolites excreted after ingestion of tryptophan by vitamin-deficient animals. The present paper describes a continuation of this work with an elaboration of the technique. The effects of deficiencies of thiamine and biotin have been investigated. Thiamine deficiency has been known for some time to decrease the conversion of tryptophan into nicotinic acid derivatives by the rat (Junqueira & Schweigert, 1948), but there has been no evidence to show at what stage in the conversion thiamine functions. A preliminary survey of other B-vitamins suggested that pantothenic acid, nicotinic acid and biotin had no effect on tryptophan metabolism

in the rat. This agreed with the known lack of effect of pantothenic acid on nicotinic acid formation from tryptophan by the rat (Junqueira & Schweigert, 1948). Moreover, should nicotinic acid, which is derived from tryptophan, be closely linked with tryptophan metabolism, it might be expected that a nicotinic acid deficiency once established should undergo an auto-intensification, which has never been observed. However, Shanmuga Sundaram, Tirunarayanan & Sarma (1954) have recently claimed that biotin is concerned in an early stage of tryptophan metabolism in *Neurospora*. The effect of biotin deficiency on tryptophan metabolism in the rat was therefore reinvestigated under more rigorous conditions. The work described in this paper has been the subject of two preliminary communications (Dalgliesh, 1954, 1955*b*).

METHODS

Animals. White rats, recently weaned, were obtained as required from the stock of the Postgraduate Medical School. These were kept throughout in wire-bottomed metabolism cages to prevent coprophagy. Diet was fed *ad lib.* except when tryptophan was administered. For this purpose, DL-tryptophan (usually approx. 1 mg./g. body wt./day for 1 or 2 days) was mixed in about half the usual amount of diet, in order to encourage complete consumption. The drop in weight, frequently observable in the growth curves after feeding tryptophan, is probably to be attributed to the temporary restriction of food rather than to any effect of the tryptophan *per se* though the palatability may be somewhat reduced. Water was freely available at all times. Acetic acid was added as preservative to the urine receivers.

Examination of metabolites. This was carried out by methods reported in detail elsewhere (e.g. Dalgliesh & Tekman, 1953; Dalgliesh, 1955*a*). Briefly, the urine was centrifuged, metabolites in the supernatant were adsorbed on deactivated charcoal, eluted with aqueous phenol, concentrated in a vacuum with simultaneous removal of phenol; the residues were submitted to paper chromatography, using butanol–acetic acid as solvent, and suitable colouring reagents applied (Dalgliesh, 1952, 1955*a*).

Thiamine-deficient regimes. Diet 1. Casein (ethanol-extracted for 3 days), 10%; sucrose, 36%; starch, 40%; fat mixture (4 parts arachis oil to 1 part cod liver oil, w/w), 10%; salt mixture (cf. Dalgliesh, 1952), 4%. The following vitamins were added to each kg. diet for the control animals [in the deficient groups the appropriate vitamin(s) were omitted]: thiamine, 10 mg.; riboflavin, 10 mg.; pyridoxine, 10 mg.; nicotinic acid, 10 mg.; *p*-aminobenzoic acid, 25 mg.; Ca pantothenate, 50 mg.; inositol, 100 mg.; folic acid (pteroylglutamic acid), 2 mg.; biotin, 0·1 mg.; vitamin K (Synkavit, Roche Products Ltd.), 1 mg. Each day 50 mg./rat of choline chloride were mixed in the diet as a 5% (w/v) solution. Each rat was individually given daily by dropper a solution of vitamin B_{12} (Cytamen, Glaxo Laboratories Ltd.) equivalent to 0·05 μg./rat/day, and also 0·1 ml./day of a 1% (w/v) solution of α-tocopherol in arachis oil.

Diet 2. This was as for Diet 1, except that the amounts/kg. diet of certain vitamins were altered to the following: riboflavin, 40 mg.; nicotinic acid, 100 mg. Choline was decreased to 5 mg./rat/day.

Diet 3. This was as for Diet 1, except that B-vitamins were given in solution by stomach tube instead of being mixed in the diet. Stock solutions were made up as follows: thiamine, 45 mg./15 ml. 0·01 N-HCl; pyridoxine hydrochloride, 36 mg./15 ml. 0·01 N-HCl; biotin, 1 mg./10 ml. 50% (v/v) ethanol; Ca pantothenate, 326 mg./100 ml. water; *p*-aminobenzoic acid, 1 g./200 ml. 5% (v/v) ethanol; a mixed solution of 1 g. inositol, 1 g. nicotinic acid and 3 g. choline chloride in 200 ml. 0·1 N-HCl; folic acid, 5 mg./100 ml. 50% (v/v) ethanol. The vitamin mixture for administration by stomach tube was made by adding 20 mg. riboflavin to a mixture of stock solutions as follows: *p*-aminobenzoic acid, 50 ml.; inositol–nicotinic acid–choline, 50 ml.; thiamine, 1·6 ml.; pyridoxine, 2·8 ml.; pantothenic acid, 16·6 ml.; biotin, 1 ml.; folic acid, 20 ml. The mixture was then made up to 500 ml. with water and stored in the cold and dark; 1 ml./day was given to each rat. Vitamin K (Synkavit) and α-tocopherol were given in arachis oil from a dropper (0·1 ml./rat/day containing 1 mg. α-tocopherol and 1 μg. Synkavit). Appropriate vitamin(s) were omitted from the deficient groups.

Biotin-deficient regime. The ethanol-extracted casein of Diet 3 was replaced by an equal weight of dried egg albumin (G. T. Gurr Ltd., London, S.W. 6). To the solid diet, before mixing to a thick paste with water, was added 1% (w/w) succinylsulphathiazole. The vitamin mixtures were the same as in Diet 3, except that the quantity of folic acid was doubled, and appropriate vitamin(s) were omitted from the deficient groups.

RESULTS

During this work various basal vitamin mixtures have been tried, and two modes of vitamin administration compared. In previous work (Dalgliesh, 1952; Charconnet-Harding *et al.* 1953) vitamins have been mixed in the diet. It was suggested to the author by Dr A. M. Copping that more satisfactory results were obtainable if vitamins were given daily in solution by stomach tube. The amount of vitamin consumed is then known with more certainty and, as no allowance need be made for incomplete consumption of food, there is useful economy. The comparisons made in this work have convinced the author that there are considerable advantages in Dr Copping's technique.

Five experiments were carried out in the thiamine-deficiency investigations. All gave the same results, described below, from the point of view of tryptophan metabolism, but growth curves for only two of these experiments are reproduced. Fig. 1 shows curves obtained in an experiment with Diet 1, and illustrates a hazard sometimes encountered when vitamins are mixed in the diet. It will be seen that, after 25 days, growth of the control animals markedly deteriorated. Satisfactory growth of control animals had previously

been obtained on a similar diet, and the work of Kandutsch & Baumann (1953) and Rombouts (1953) on the decomposition of thiamine in laboratory diets makes it probable that the poor growth of the control rats shown in Fig. 1 was due to a thiamine deficiency. In case a vitamin imbalance was involved, another group of animals were given Diet 2, in which the proportions of the vitamins were altered. Growth of the control animals was now comparable to that found for black-and-white rats by Copping, Crowe & Pond (1951), who gave their vitamins by stomach tube, but the net consumption of vitamins on Diet 2 was much higher than in the experiments of Copping *et al.* It seemed likely that thiamine decomposition and possible similar complications would be less likely to arise when using Dr Copping's technique, described under Diet 3. Growth curves obtained on such a regime are shown in Fig. 2. Growth of the control animals was more satisfactory than when the vitamins were mixed in the diet; moreover, once practice had been obtained, administration of vitamins by stomach tube was quicker and less troublesome, the animals easily becoming accustomed to the procedure. Control animals on regimes having the vitamins mixed in the diet showed a higher excretion of tryptophan metabolites after supplementary tryptophan than did animals on a stock diet or those receiving their vitamins by stomach tube, suggesting that in the former case the vitamin intake was less balanced.

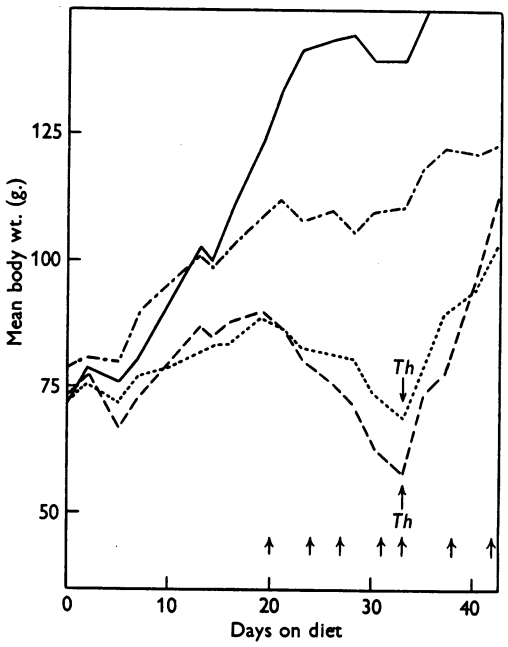


Fig. 1. Growth curves of control (——), thiamine-deficient (– – –), pyridoxine-deficient (·–·–·) and thiamine–pyridoxine-deficient (.....) female rats fed by the vitamins-in-the-diet technique. Each curve is the mean for three animals. Thiamine was given to the deficient animals from the points marked *Th*. Small arrows at the bottom represent supplementary tryptophan in the diet.

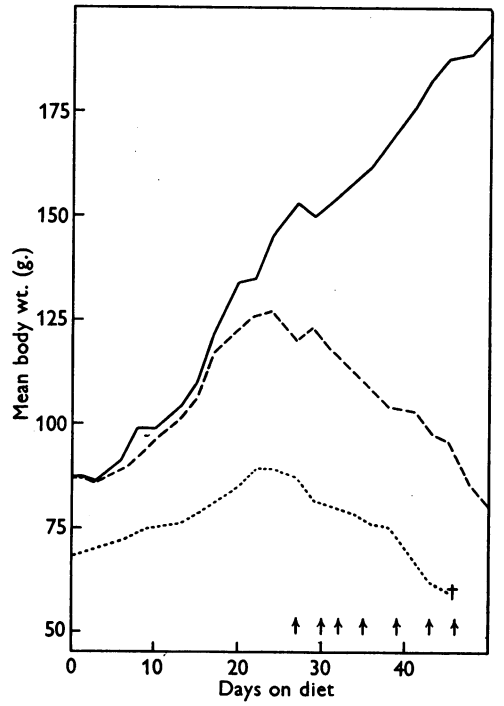


Fig. 2. Growth curves of control (——), thiamine-deficient (– – –) and thiamine–pyridoxine-deficient (.....) female rats given vitamins by the stomach-tube technique. Each curve is the mean for five animals. Small arrows at the bottom represent supplementary tryptophan in the diet. Animals had to be killed at the point marked †.

The results of feeding tryptophan to the various groups of animals were the same in all experiments. At the tryptophan dose level used, the control animals normally excreted small but detectable quantities of tryptophan metabolites. Tryptophan administration was generally started about 3 weeks after initiation of the dietary regime. At this stage excretion by the thiamine-deficient and control rats was indistinguishable, and the rats deficient in thiamine and pyridoxine showed a typical pyridoxine-deficient excretory pattern. As thiamine deficiency became established, as shown by the growth curves, the small excretion of tryptophan metabolites by the tryptophan-fed thiamine-deficient rats ceased. Simultaneously, in the rats

deficient in thiamine and pyridoxine, excretion of the metabolites typical of pyridoxine deficiency decreased and, by the time the animals approached their starting weights, metabolite excretion was confined to a relatively small output of xanthurenic acid. However, kynurenine fed at this stage resulted in a large output of metabolites typical of pyridoxine deficiency. There was no evidence of formylkynurenine excretion on giving tryptophan to animals with a simple thiamine deficiency. On giving thiamine to deficient animals there was a dramatic growth response, as shown in Fig. 1. Simultaneously, the typical pyridoxine-deficient excretory pattern returned in those animals previously doubly deficient.

Typical excretory patterns after establishing both thiamine and pyridoxine deficiencies are summarized in Table 1. It should be emphasized that the metabolites under discussion in this paper are those related to transformation of tryptophan into nicotinic acid. All animals, deficient or otherwise, described in this paper excreted after tryptophan administration appreciable amounts of indolic compounds. The major ones were tryptophan, indican, and two spots moving fast in butanol–acetic acid, the slower moving of which is considered to be principally indole-β-aceturic acid and the faster moving indoleacetic acid, together with indole derived from faecal contamination of the urine. In the biotin-deficiency experiments, in which succinylsulphathiazole was fed, the indole-aceturic acid spot was smaller than in the other experiments. The indole–indoleacetic acid spot was less markedly decreased.

Several diets were tried in the biotin-deficiency experiments. Biotin-deficient casein-based diets with added aureomycin (vitamins mixed in the diet) gave a slight deficiency as judged by growth curves, but the degree of deficiency was considered quite inadequate for the investigation. Diets with egg albumin (containing the biotin antagonist avidin) as protein source were tried by both the vitamins-in-the-diet and vitamins-by-stomach-tube techniques. Somewhat greater deficiencies were obtained, but again these were considered inadequate. By combining supplementary succinylsulphathiazole with a diet based on egg albumin, marked deficiencies were obtained readily. These results contrast with those of Emerson & Wurtz (1944), who found an egg-albumin diet to be adequate to produce a high degree of biotin deficiency, which was not increased by succinylsulphathiazole. The amounts of succinylsulphathiazole metabolites excreted were not sufficient to interfere with chromatography of the tryptophan metabolites.

Growth curves obtained with the combined egg albumin–succinylsulphathiazole regime are shown in Fig. 3. The biotin-deficient group showed appreciable deficiency (thinning of hair, scale formation) after 4 weeks. Scaly dermatitis, 'spectacle eyes' and 'kangaroo gait' were apparent by the sixth week and marked after the eighth week. The biotin-deficient rats had lost almost all their hair by the tenth week and at about this stage some oedema became evident. In the group deficient in biotin and pyridoxine the loss of hair was less marked, but the dermatitis was more severe and the eyes became completely closed. The control group, differing from the biotin-deficient group only in receiving biotin, grew well throughout, and the picture was therefore not complicated by any accompanying folic acid deficiency.

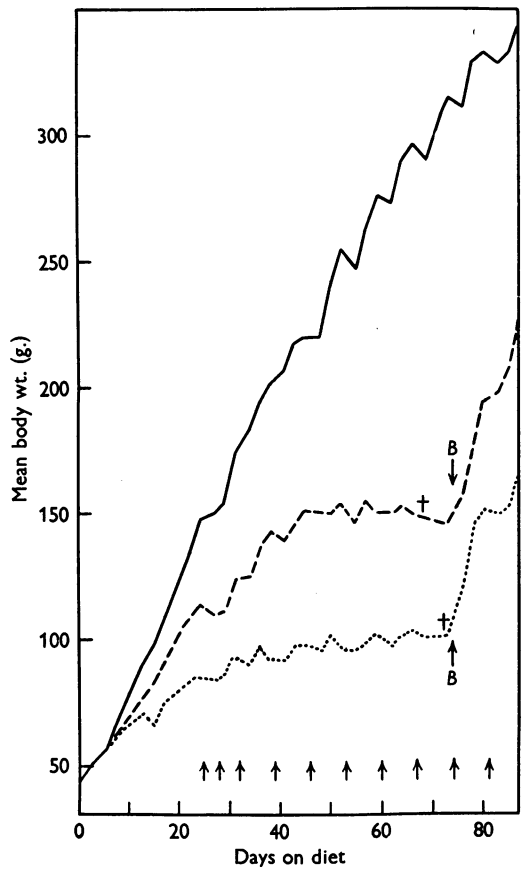


Fig. 3. Growth curves of control (——), biotin-deficient (– – –), and biotin–pyridoxine-deficient (.....) male rats fed on vitamins by the stomach-tube technique. Each curve is the mean for three animals. Small arrows at the bottom represent supplementary tryptophan in the diet. An animal died at the points marked †. Biotin was given to the deficient animals from the points marked *B*.

Table 1. *Typical metabolite-excretory patterns after feeding tryptophan to control and vitamin-deficient rats*

Excretion represented by +; metabolites sometimes excreted by ±.

Metabolite	Non-deficient	Thiamine-deficient	Pyridoxine-deficient	Thiamine–pyridoxine-deficient	Biotin-deficient	Biotin–pyridoxine-deficient
Kynurenine	±	−	+ +	±	±	+ +
Kynurenine conjugates	−	−	+ +	±	−	+ +
Hydroxykynurenine	−	−	+ +	±	−	+ +
Hydroxykynurenine conjugates	−	−	+ + +	±	−	+ + +
Xanthurenic acid (4:8-dihydroxy-quinoline-2-carboxylic acid)	+	−	+ + + +	+	+	+ + + +
Anthranilic acid	±	−	−	−	±	−
Anthranilic acid conjugates	±	−	−	−	±	−

Administration of biotin to the deficient animals caused a dramatic response in growth (Fig. 3) and a noticeable growth of hair in the denuded animals within 24 hr.

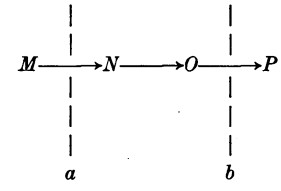
Throughout the experiment no appreciable differences could be seen in the tryptophan-metabolite excretion of biotin-deficient and control rats, and at no stage did there appear to be any change in the typical pyridoxin-deficient excretory pattern of the group deficient in biotin and pyridoxine. These results are also summarized in Table 1.

DISCUSSION

Investigations on intermediary metabolism in the intact animal can have the advantage over enzymic techniques that reactions may be revealed which are brought about by enzymes too unstable to lend themselves readily to *in vitro* investigations, and that reaction chains may be deduced from the occurrence of some intermediate which could not readily be deduced in an isolated or reconstituted system. Moreover, even if the amount and activity of an enzyme in a given organ are known, its importance in the overall picture may be difficult to assess, other than by a study of end products, as the available enzyme cannot be fully efficient unless provided with the right precursor in the right place at the right time. *In vitro* and *in vivo* experiments are thus complementary, and the remarkable recent advances in enzymic techniques do not reduce the need for experiments on the intact animal.

The approach used in these experiments is analogous for mammals to the study of deficient mutants in micro-organisms. It depends on the function of many B-vitamins as constituents of coenzymes. In a deficiency of such a vitamin an enzymic reaction for which it provides a coenzyme may be inhibited. If one reaction in a metabolic chain is inhibited and a substance prior to it in the chain is administered to the animal, it is to be expected that the substrate of the inhibited reaction (and/or simple derivatives) may be excreted. Such an approach has led to useful information on the function of pyridoxine (Dalgliesh, 1952) and riboflavin (Charconnet-Harding *et al.* 1953) in tryptophan metabolism. The method, of course, has limitations. Thus if a vitamin deficiency results in a definite change in a pattern of metabolite excretion, definite conclusions may be drawn. But if there is deficiency, as shown perhaps by the growth curve, but no change in the pattern of metabolite excretion, it cannot be concluded that that vitamin is not concerned in that metabolic chain. The coenzyme in which the vitamin participates may be concerned in many reactions involving enzymes which have widely differing affinities for the coenzyme. In a deficiency, these reactions are not necessarily inhibited to the same degree. For example, pyridoxal phosphate is the coenzyme in transamination reactions among many others, but transamination can be apparently unaffected by a degree of vitamin B_6-deficiency which would result in marked inhibition of other vitamin B_6-dependent enzymes (cf. Snell, 1953).

An improved technique is described in this paper which is useful in certain cases. If we have a metabolic chain and a block occurs at stage *b*, then



excretion of *O* after administration of *M* has obvious significance. But it is not necessarily justifiable to deduce from an absence of metabolite excretion that there is a block at *a*. However, if blocks at *a* and *b* are combined, formation of *O* should be inhibited by block *a*, so that the excretion of *O* previously observed with a simple block at *b* should no longer occur. Thus the reality of a block at *a* can be checked.

The general outline of the pathway for conversion of tryptophan into nicotinic acid is summarized in Fig. 4 (for collected evidence see review by Dalgliesh, 1955*c*). Pyridoxine is concerned at the stage marked *C* ... *C* (Dalgliesh, 1952) and riboflavin probably at the stage marked *B* ... *B* (Charconnet-Harding *et al.* 1953). The present results show that thiamine deficiency inhibits formation of the substrate of the pyridoxine-dependent reaction. It must therefore function in the conversion of tryptophan into formylkynurenine, stage *A* ... *A* in the diagram, or in the formation of kynurenine from formylkynurenine. The latter is inherently less probable and is excluded by the absence of formylkynurenine excretion by the thiamine-deficient animals.

The results obtained in biotin deficiency strongly suggest that in the rat biotin does not influence any stage in the conversion of tryptophan into hydroxykynurenine. The results thus disagree with the conclusions of Shanmuga Sundaram *et al.* (1954) on the function of biotin in tryptophan metabolism in *Neurospora*, in which they consider biotin to be a cofactor in formylkynurenine formation. There remains, however, the possibility, considered unlikely, that biotin is part of a coenzyme which is not readily dissociated from its enzyme in biotin deficiency in the rat. The Indian workers based their results on experiments with a vitamin antagonist, which implies that any such coenzyme is dissociable in *Neurospora*.

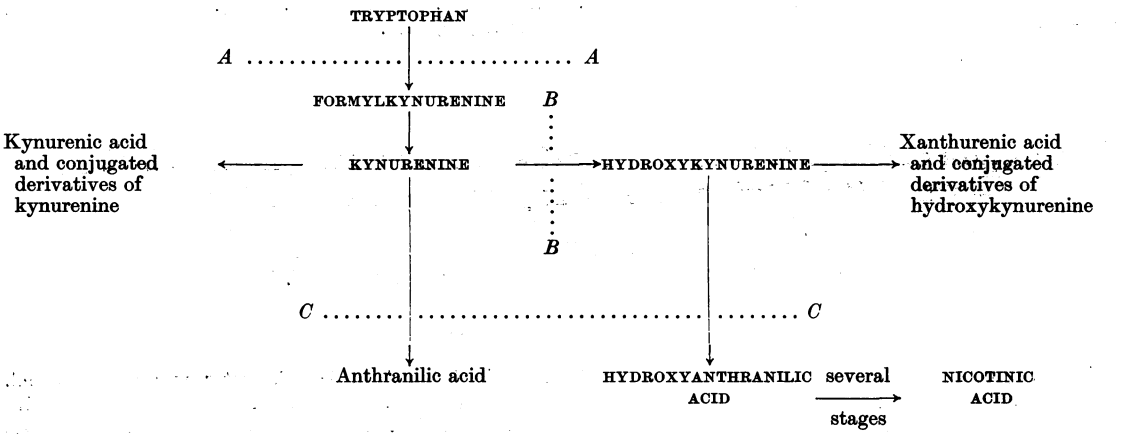


Fig. 4. Outline of the pathway for conversion of tryptophan into nicotinic acid. Intermediates on the main pathway in small capitals, by-products in ordinary type.

These experiments thus establish that thiamine influences tryptophan metabolism at stage *A* ... *A*, but they do not show whether or not the effect is direct, i.e. whether thiamine is functioning as part of an enzyme cofactor. The reaction involved is quite different in type from the reactions normally considered to be dependent on thiamine. But it is of some interest in this connexion that the two vitamin antagonists oxythiamine and neopyrithiamine have very different actions. Oxythiamine inhibits pyruvate decarboxylation without producing marked neurological changes, whereas neopyrithiamine produces the neurological symptoms without necessarily affecting pyruvate decarboxylation (Woolley & Merrifield, 1954). This strongly suggests participation of thiamine in reactions other than α-keto acid decarboxylation, normally considered to be its prime function.

The inhibition in thiamine deficiency of the first stage of tryptophan metabolism by the kynurenine–nicotinic acid pathway may assist in the study of other pathways by forcing metabolism to occur by alternative routes. There is of course no evidence that thiamine deficiency does not also affect these other pathways, and a complication may also arise if an increased blood α-keto acid level causes an abnormal degree of indolepyruvic acid formation by transamination.

SUMMARY

1. The metabolism of tryptophan has been studied in rats deficient in (*a*) thiamine, (*b*) thiamine and pyridoxine, (*c*) biotin, and (*d*) biotin and pyridoxine.

2. On imposing a thiamine deficiency on pyridoxine-deficient rats, and feeding supplementary tryptophan, the metabolite-excretory pattern typical of pyridoxine deficiency becomes abolished or much reduced. Formation of the substrates of the kynureninase reaction is therefore inhibited. Kynurenine fed at the same stage results in a marked metabolite excretion. No formylkynure-

nine is excreted on giving tryptophan to rats with a simple thiamine deficiency.

3. It is therefore concluded that thiamine is concerned in the conversion of tryptophan into formylkynurenine.

4. Similar experiments combining biotin and pyridoxine deficiencies suggest that biotin is unlikely to be concerned in any stage in the conversion of tryptophan into hydroxykynurenine.

5. Considerable advantages were found in giving vitamins by stomach-tube rather than by mixing them in the diet.

I thank Dr A. M. Copping for her advice, and Mr A. Asatoor and Miss R. Paul for skilled assistance.

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Excretion of Conjugated 2-Amino-3-Hydroxyacetophenone by Man, and its Significance in Tryptophan Metabolism

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In a survey of aromatic metabolites in the urine of patients with various leukaemias and anaemias it was noticed that several of these urines contained appreciable amounts of a substance with a strong pale-blue fluorescence. This paper describes its identification.

METHODS

Aromatic metabolites were isolated from the urines by adsorption on deactivated charcoal and subsequent phenol elution, as described in detail elsewhere (Dalgliesh, 1955*a*). Preparative chromatograms were run by the descending technique on Whatman 3 MM paper and other chromatograms similarly on Whatman no. 1 paper. Paper electrophoresis was carried out for 45–60 min. on Whatman no. 1 paper soaked in 0·4% (v/v) aqueous acetic acid with an applied potential gradient of 50 v/cm. Ultraviolet absorption spectra of eluates were determined with a Unicam spectrophotometer model SP. 500. The reagents used for determining reactive groups have been described elsewhere (Dalgliesh, 1955*a*).

The following solvents were used for chromatography: the organic phase of a *n*-butanol–glacial acetic acid–water mixture (4:1:5 by vol.); 'Phenol liquefactum' B.P.–water, 100:15 (v/v) ('neutral phenol'); *n*-pentanol–pyridine–water (7:7:6 by vol.); aqueous KCl (20%, w/v); aqueous sodium formate (5%, w/v).

Persulphate oxidation of o-aminoacetophenone (cf. Boyland & Sims, 1954). To *o*-aminoacetophenone hydrochloride (70 mg.) in 1·5 ml. water was added 0·9 ml. N-NaOH (approx. 2·2 equiv.) and sufficient acetone to give a clear solution. A solution of potassium persulphate (110 mg., 1 equiv.) in 10 ml. water was added, and the mixture set aside overnight. The brown reaction product was then diluted, filtered and acidified with acetic acid, and the products were adsorbed on deactivated charcoal. This was then eluted with phenol and the products were purified and examined by the procedures used for urinary metabolites.

RESULTS

The unknown substance had a fluorescence very similar in colour to that of kynurenine. It moved on butanol–acetic acid chromatograms at a medium R_F slightly faster than, but usually overlapping, indican, and in its turn slightly slower than, but overlapping, a non-fluorescent substance, positive to Ehrlich's reagent (*p*-dimethylaminobenzaldehyde in dilute HCl), which will be the subject of a future communication. Preparative chromatography successively in butanol–acetic acid, neutral phenol and again in butanol–acetic acid, the appropriate areas being cut out and eluted at each stage, gave an extract in which no other substances could be detected chromatographically. The ultraviolet spectrum of the final eluate showed maxima at about 260 and 360 mμ., characteristic of a